



Functional characterization of the human intestinal NaPi-IIb cotransporter in hamster fibroblasts and *Xenopus* oocytes

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Abstract

The recently cloned NaPi-IIb cotransporter is an apical membrane protein that is involved in the absorption of phosphate in the intestine. To expedite functional and structural studies, the human intestinal NaPi-IIb cotransporter was stably expressed in hamster fibroblast (PS120) cells. The hNaPi-IIb cDNA stably transfected cells exhibited a 1.8-fold higher sodium-dependent phosphate uptake than vector DNA transfected cells, and had a K_m for Pi of $\sim 106 \mu\text{M}$ and a K_m for Na^+ of $\sim 34 \text{ mM}$. The hNaPi-IIb cotransporter was also expressed in *Xenopus* oocytes and it exhibited a K_m for Pi of $\sim 113 \mu\text{M}$ and a K_m for Na^+ of $\sim 65 \text{ mM}$. The hNaPi-IIb cotransporter expressed in both PS120 cells and oocytes was inhibited by high external pH. Furthermore, phosphate uptake mediated by the hNaPi-IIb cotransporter was inhibited by 5 mM phosphonoformic acid (PFA), 1 mM arsenate and 100 nM phorbol myristate acetate (PMA). These results demonstrate that the human intestinal NaPi-IIb cotransporter is functional when expressed in hamster fibroblasts, and that this model system may be useful in the future to identify NaPi-IIb cotransporter-specific inhibitors.

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Keywords: Type IIb sodium-phosphate cotransporter; PS120 cell; *Xenopus* oocyte

1. Introduction

Phosphate (Pi) plays a critical role in the body as a constituent of bone and tooth for body development, and as a urinary buffer for pH in body acid–base balance regulation. Pi also plays a major role in cellular metabolism. Sodium-coupled phosphate transport is the major form of Pi absorption in the intestine, which has been studied in human [1], rat [2–4], rabbit [5] and mouse [6]. We have previously cloned and described a human intestinal sodium-dependent phosphate (hNaPi-IIb) cotransporter [7]. Functional characterization of this cotransporter by expression in *Xenopus* oocytes and analysis of NaPi-IIb mRNA expression suggested that this protein is involved in intestinal sodium-dependent phosphate absorption [7]. Further studies showed

that the intestinal NaPi-IIb cotransporter is regulated by hormones involved in the modulation of intestinal nutrient absorption including epidermal growth factor (EGF) [8], 1,25 (OH)₂ vitamin D₃ [9] and glucocorticoids [10]. These studies exemplified the potential importance of this transporter in intestinal phosphate absorption. These previous investigations explored the regulation of NaPi-IIb cotransporter gene expression. However, extensive functional characterization of the human NaPi-IIb cotransporter has not yet been performed.

PS120 cells are fibroblast cells derived from the Chinese hamster [11]. This cell line has been used to functionally express and characterize several intestinal transport proteins [12–19]. Functional expression of the human NaPi-IIb cotransporter in a mammalian cell expression system would allow one to perform further biological analyses, such as membrane topology and function/structure studies, antiserum characterization, and studies designed to characterize the functional cotransporter regulation.

In this communication, we demonstrate that the human NaPi-IIb protein can be functionally expressed in PS120 cells. We further show that hNaPi-IIb cotransporter activity

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in PS120 cells exhibits similar transport kinetics as compared to the oocyte expression system. Thus, the PS120 cell expression system will allow for investigation of hNaPi-IIb cotransporter structure/function relationships and functional regulation, and will further allow for the identification of specific inhibitors of the hNaPi-IIb protein.

2. Materials and methods

2.1. Plasmid construction

The entire human NaPi-IIb cDNA was isolated from the original cloning vector, EAK8, by *NotI* and *EcoRI* digestion [7]. The isolated insert was then ligated into the mammalian expression vector pTarget (Promega, Madison, WI). This vector allows for the expression of the cDNA in mammalian cells under the control of the CMV promoter. Clones containing the hNaPi-IIb cDNA insert were confirmed by DNA sequencing. The final construct contained the hNaPi-IIb cDNA, which included the full-length coding region flanked by 35 bp of 5'-untranslated sequence and 2028 bp of 3'-untranslated sequence. Plasmid DNA used in subsequent studies was purified with the Qiagen DNA purification kit (Qiagen, Valencia, CA).

2.2. Cell culture

PS120 cells were a gift from Dr. Pouyssegur (University of Nice, Nice, France). They are fibroblast-derived cells isolated from the lung of the Chinese hamster [11]. Cells were cultured at 37 °C in a 95% air–5% CO₂ atmosphere in DME medium supplemented with 1% penicillin–streptomycin and 10% fetal bovine serum (FBS). Cells were passaged every 48 h. Media and other reagents used for cell culture were purchased from Irvine Scientific (Irvine, CA).

2.3. Stable transfection

PS120 cells were cultured in 35 mm plates. When cell density reached 60–70%, liposome-mediated transfection was performed. Plasmid DNA (1.0 µg) (pTarget plus hNaPi-IIb cDNA or empty pTarget vector), and 5 µl lipofectamine (Gibco/BRL, Grand Island, NY) were mixed with 400 µl Opti-MEM medium (Gibco/BRL) for 30 min at room temperature. The mixture was then added to the cells and they were incubated for 5 h, after which an equal volume of medium containing 20% FBS was added and the cells were incubated overnight. The next morning, the medium was removed and replaced with standard medium containing 10% FBS. After 48 h, the medium was replaced with G418 medium (standard medium with 10% FBS plus G418 (800 µg/ml)). Transfected cells were cultured in G418 medium for 7–10 days to eliminate nontransfected cells before experiments were performed.

2.4. PCR analysis to detect NaPi-IIb expression in PS120 cells

mRNA was purified from vector or human NaPi-IIb cDNA transfected PS120 cells using the FastTrack mRNA Isolation Kit (Invitrogen, Carlsbad, CA). RT-PCR was performed as previously described [8]. Primers used to detect the NaPi-IIb transcript were designed from the human NaPi-IIb cDNA (GenBank accession #AF146796). The forward primer was at 1264–1283 bp (5'-TTGCATGGTT-GACTGGCTAC-3'), and the reverse primer was at 1793–1812 bp (5'-GCAGGAAGTTCCAGTTCTGG-3'). The expected amplicon size was 548 bp.

2.5. Phosphate uptake measurements

Stably transfected PS120 cells were grown on 24-well plates in the presence of G418, and phosphate uptake was performed when cells were 95% confluent. Cells were washed with 1.5 ml uptake buffer twice (137 mM NaCl or choline Cl, 5.4 mM KCl, 2.8 mM CaCl₂, 1.2 mM MgSO₄, 10 mM Hepes/Tris, pH 7.4) and incubated with 300 µl uptake buffer plus 0.1 mM PO₄ and 1 µCi/ml ³²P-labeled PO₄. After various times of incubation at 25 °C, the uptake solution was removed and cells were washed three times with 1 ml ice-cold stop solution (137 mM NaCl, 10 mM Hepes/Tris, pH 7.4). Cells were then lysed in 200 µl 1 × passive lysis buffer (PLB) (Promega), and aliquots were used to measure radioactivity by liquid scintillation counting and to determine protein concentration by BCA protein assay reagent kit (Pierce, Rockford, IL).

For determining the apparent K_m for Pi, extracellular Pi concentration was varied between 10 µM and 1 mM, while the pH was kept constant at 7.4. For testing the K_m for Na, extracellular Na concentration was varied between 10 and 140 mM while pH remained constant at 7.4. The Tris/Hepes buffer system was used throughout to produce pHs between 6.0 and 8.5. After stable transfection, cells from passage five to eight were used for the above studies. The incubation time point was chosen in the linear portion of the time course curve.

For inhibition studies, various inhibitors known to inhibit other NaPi isoforms were used. The incubation time point was chosen to be close to the saturation portion of the time course curve. The chemical concentrations used for these studies were: 100 nM for phorbol myristate acetate (PMA), 5 mM for phosphonoformic acid (PFA), and 1 mM for arsenate. All these chemicals were purchased from Sigma (St. Louis, MO).

For the calculation of sodium-dependent phosphate absorption, phosphate uptake in the absence of sodium was removed from uptake in the presence of sodium.

For the calculation of human NaPi-IIb protein-mediated phosphate absorption, total sodium-dependent phosphate uptake in vector DNA transfected PS120 cells was sub-

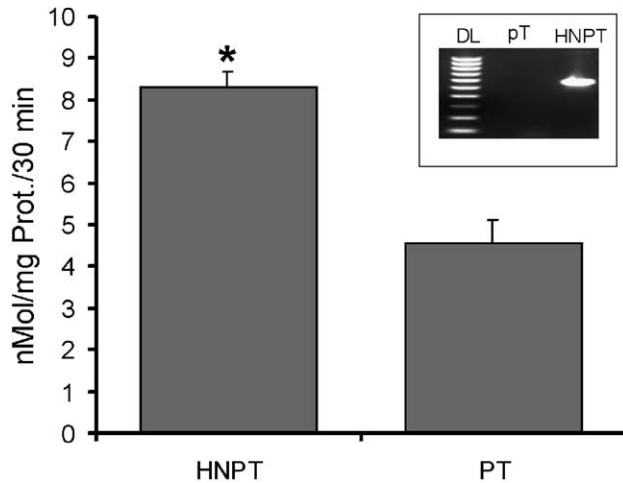


Fig. 1. Characterization of hNaPi-IIb cotransporter expression in PS120 cells. Sodium-dependent phosphate uptake in transfected PS120 cells is depicted. The values shown are mean \pm S.E. ($n=6$; $*P<0.003$). The inset shows hNaPi-IIb mRNA expression in transfected PS120 cells by RT-PCR. (pT): vector DNA transfected cells. (HNPT): hNaPi-IIb cDNA transfected cells. (DL): 100 bp DNA ladder.

tracted from total sodium-dependent phosphate uptake in hNaPi-IIb cDNA transfected PS120 cells.

2.6. Functional characterization of the human intestinal NaPi-IIb cotransporter in oocytes

Initially, *Xenopus laevis* frogs (NASCO) were anesthetized in ice water with 0.05% benzocaine for 15 min.

Oocytes were then dissected from the frogs and defolliculated, as described previously [20]. Oocytes were maintained at 18 °C in Barth's solution containing 50 mg/ml gentamycin sulfate, 2.5 mM sodium pyruvate, and 5% heat-inactivated horse serum. The oocytes were injected with 50 ng of human intestinal NaPi-IIb cRNA 1 day following isolation. Transport of $\text{KH}_2^{32}\text{PO}_4$ (DuPont-NEN) was measured 3 days after injection as previously described [21]. Oocyte uptake buffers consisted of: 100 mM NaCl (Na buffer) or 100 mM choline chloride (choline buffer), with 2 mM KCl, 1 mM CaCl_2 , 1 mM MgCl_2 , and 10 mM Hepes/Tris, pH 7.5. For transport assays, oocytes were washed briefly in choline buffer to remove serum and then incubated in 0.4 ml of uptake buffer. After the indicated time period, uptake was stopped with four 4-ml washes of ice-cold choline buffer. Individual oocytes were transferred to scintillation vials and dissolved in 0.5 ml 10% SDS, and the radioactivity was measured by scintillation counting. Counts in control (uninjected) oocytes were subtracted from counts in cRNA-injected oocytes. All experiments were performed in 4–10 injected oocytes.

2.7. Curve fit and statistical analysis

All curves, including calculation of kinetic constants, were done by nonlinear regression using the SigmaPlot program (Jandel Scientific). ANOVA Post Hoc Tests (Stat-View 5.0.1 version, SAS Institute, Cary, NC) were used to compare values of the experimental data. P values of <0.05 were considered significant.

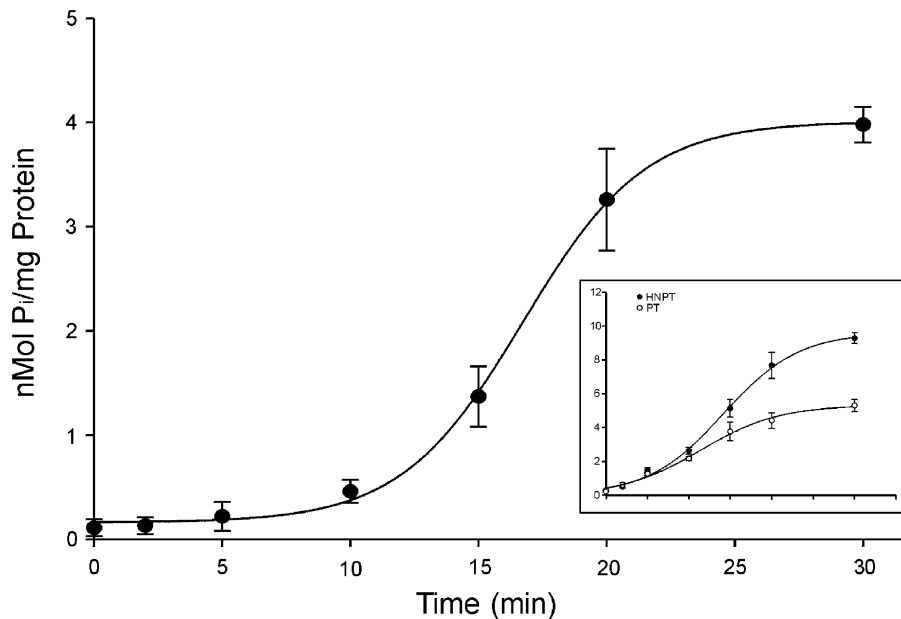


Fig. 2. Time course of sodium-dependent phosphate uptake mediated by hNaPi-IIb cotransporter in PS120 cells. Cells were incubated in uptake solution for up to 30 min. Phosphate was added at a final concentration of 0.1 mM. The values shown are mean \pm S.E. ($n=5$). The inset shows sodium-dependent phosphate uptake in vector DNA (pT) or human NaPi-IIb cDNA (HNPT) transfected cells. The axis labels in the inset are the same as in the figure.

3. Results

3.1. Characterization of human NaPi-IIb expression in transfected PS120 cells

PS120 cells were transfected with vector DNA or vector plus hNaPi-IIb cDNA by a liposome-mediated method. Transfected cells were propagated in G418-containing medium. Expression of the hNaPi-IIb cotransporter in transfected PS120 cells was detected by sodium-dependent phosphate uptake and RT-PCR. As shown in Fig. 1, a 2-

fold increase in sodium-dependent phosphate absorption was observed from the hNaPi-IIb cDNA transfected PS120 cells when compared with vector DNA transfected PS120 cells. hNaPi-IIb mRNA was also detected by RT-PCR in cDNA transfected PS120 cells (Fig. 1, inset).

3.2. Pi transport in stably transfected PS120 cells

To characterize Pi transport in transfected PS120 cells, the relationship between Pi uptake rates and incubation times were investigated. PS120 cells were incubated for

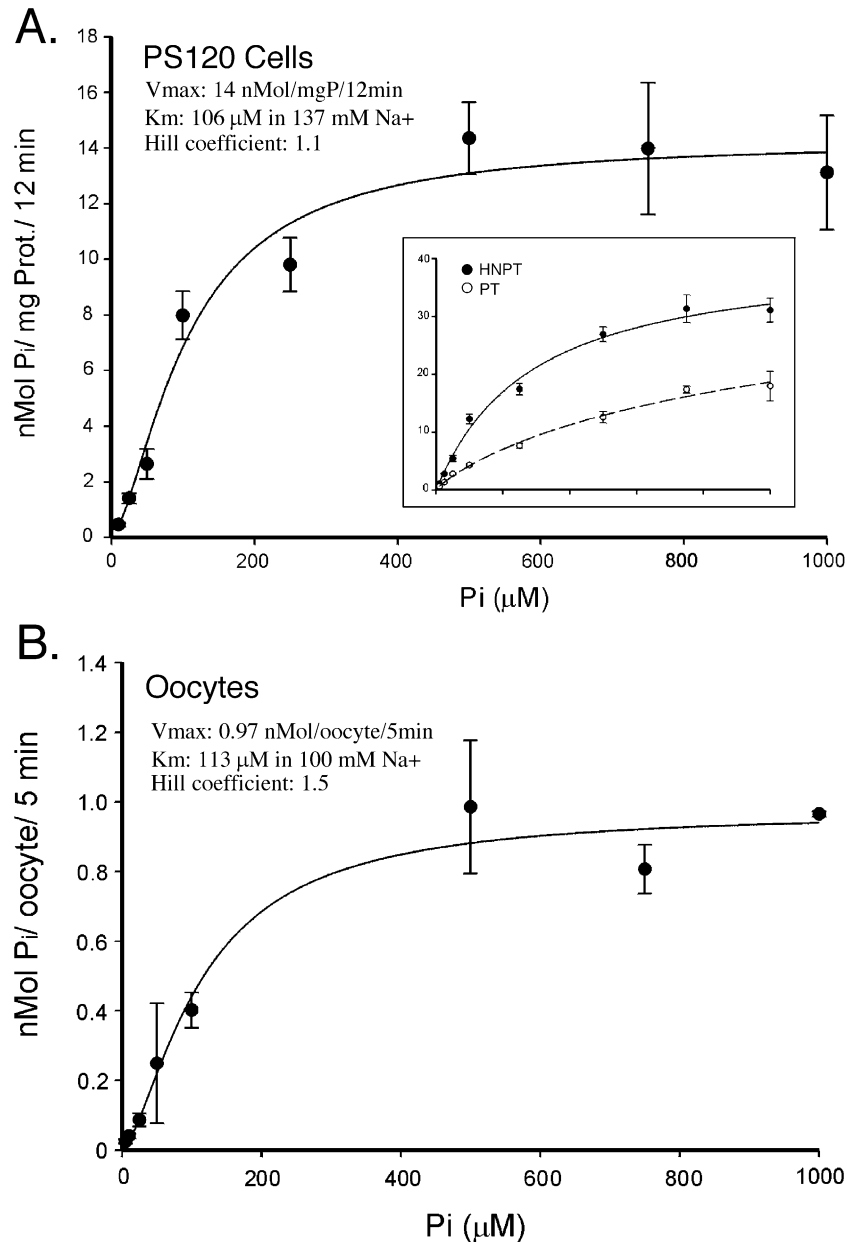


Fig. 3. Kinetics of phosphate transport. Panel A. Kinetics of phosphate transport mediated by human NaPi-IIb transporter in PS120 cells. Phosphate uptake was measured within the linear portion of the uptake curve shown in Fig. 2 (12 min) at different Pi concentrations in the presence of 137 mM NaCl. The values shown are mean \pm S.E. ($n=4$). The inset illustrates the uptake in hNaPi-IIb cDNA (●) and vector DNA (○) transfected PS120 cells. The axis labels in the inset are the same as in the figure. Panel B. Kinetics of phosphate transport in human NaPi-IIb cRNA injected oocytes. Five-minute Pi uptake with Pi concentrations, between 5 and 1000 μ M, was measured in the presence of 100 mM sodium. The values shown are mean \pm S.E. ($n=5$ oocytes).

various time periods in the presence of 100 μM Pi, and Pi absorption was measured in the presence or absence of sodium. Fig. 2 shows a time course study of sodium-dependent phosphate uptake mediated by hNaPi-IIb cotransporter in PS120 cells. As shown, hNaPi-IIb-mediated phosphate uptake increased as incubation times increased. A linear uptake rate occurred between 10 and 20 min and reached a plateau after 20 min. Sodium-independent uptake was very low (< 0.6 nmol/mg protein/30 min) in both vector

DNA and hNaPi-IIb cDNA transfected PS120 cells (data not shown).

3.3. hNaPi-IIb cotransporter phosphate uptake kinetics

To compare Pi uptake kinetics in a mammalian cell expression system and in an oocyte expression system, the hNaPi-IIb cDNA or vector DNA were transfected into PS120 cells, and hNaPi-IIb cRNA was injected into oocytes. Pi

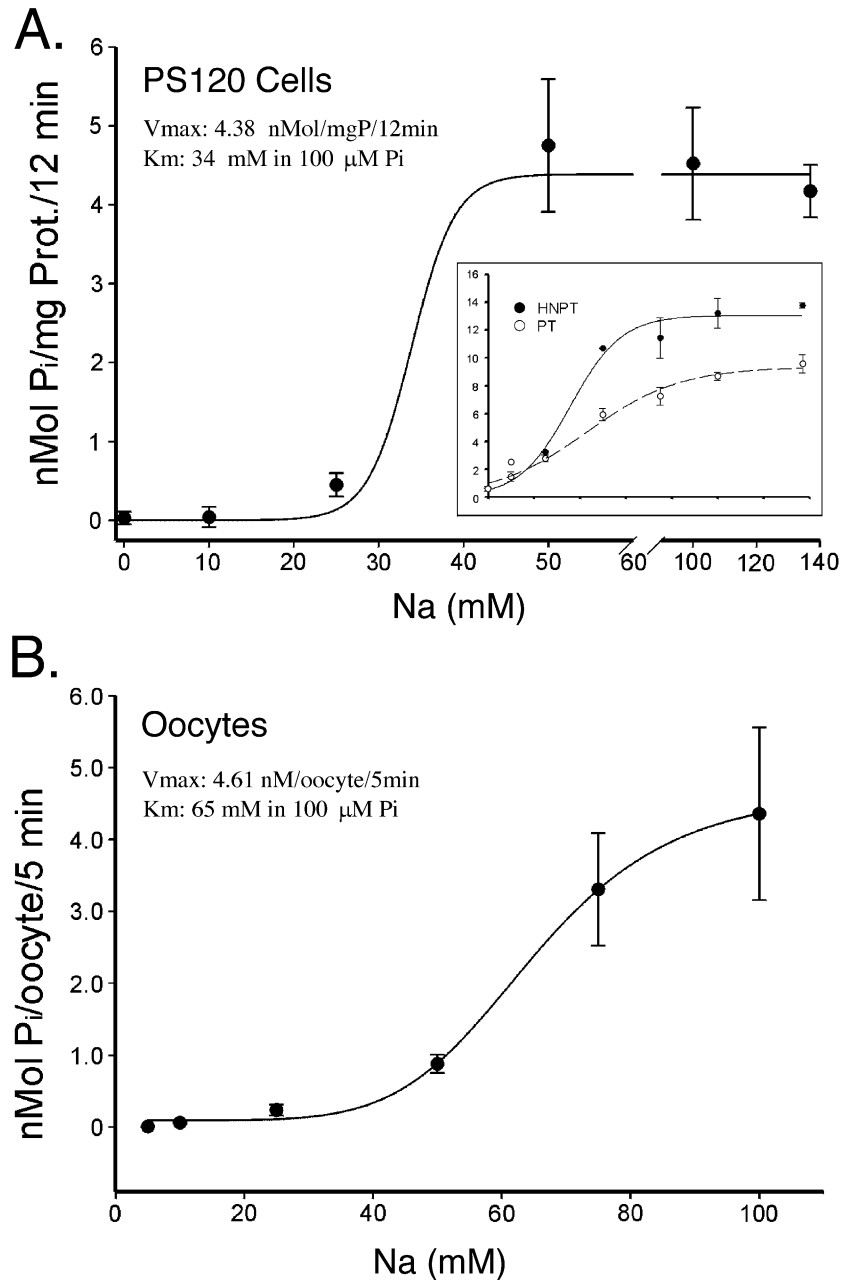


Fig. 4. Sodium activation of phosphate cotransport. Panel A. Phosphate uptake mediated by the human NaPi-IIb cotransporter in PS120 cells. Phosphate uptake was measured in the presence of 100 μM Pi under linear uptake conditions (12 min) as a function of sodium concentration. The values shown are mean \pm S.E. ($n=4$). The inset illustrates the sodium-dependent uptake in human NaPi-IIb cDNA (●) and vector DNA (○) transfected cells. The axis labels in the inset are the same as in the figure the scale of x-axis in inset is 0, 20, 40, 60, 80, 100, 140. Panel B. Phosphate transport in human NaPi-IIb cRNA injected oocytes. Five-minute uptake was measured in the presence of 0.1 mM phosphate plus various concentrations of Na. The values shown are mean \pm S.E. ($n=5$ oocytes).

uptake was measured in the presence of various phosphate concentrations at a single time point. As shown in Fig. 3, sodium-dependent phosphate transport mediated by hNaPi-IIb cotransporter was concentration-dependent and saturable in both PS120 cells and oocytes. In PS120 cells, sodium-

dependent phosphate uptake exhibited a maximal velocity (V_{\max}) of 14 nmol/mg protein/12 min, with a Michaelis–Menten constant (K_m) of 106 μ M (Fig. 3A). In oocytes, the kinetics for phosphate cotransport were $V_{\max} = 0.97$ nmol/oocyte/5 min and $K_m = 113$ μ M (Fig. 3B).

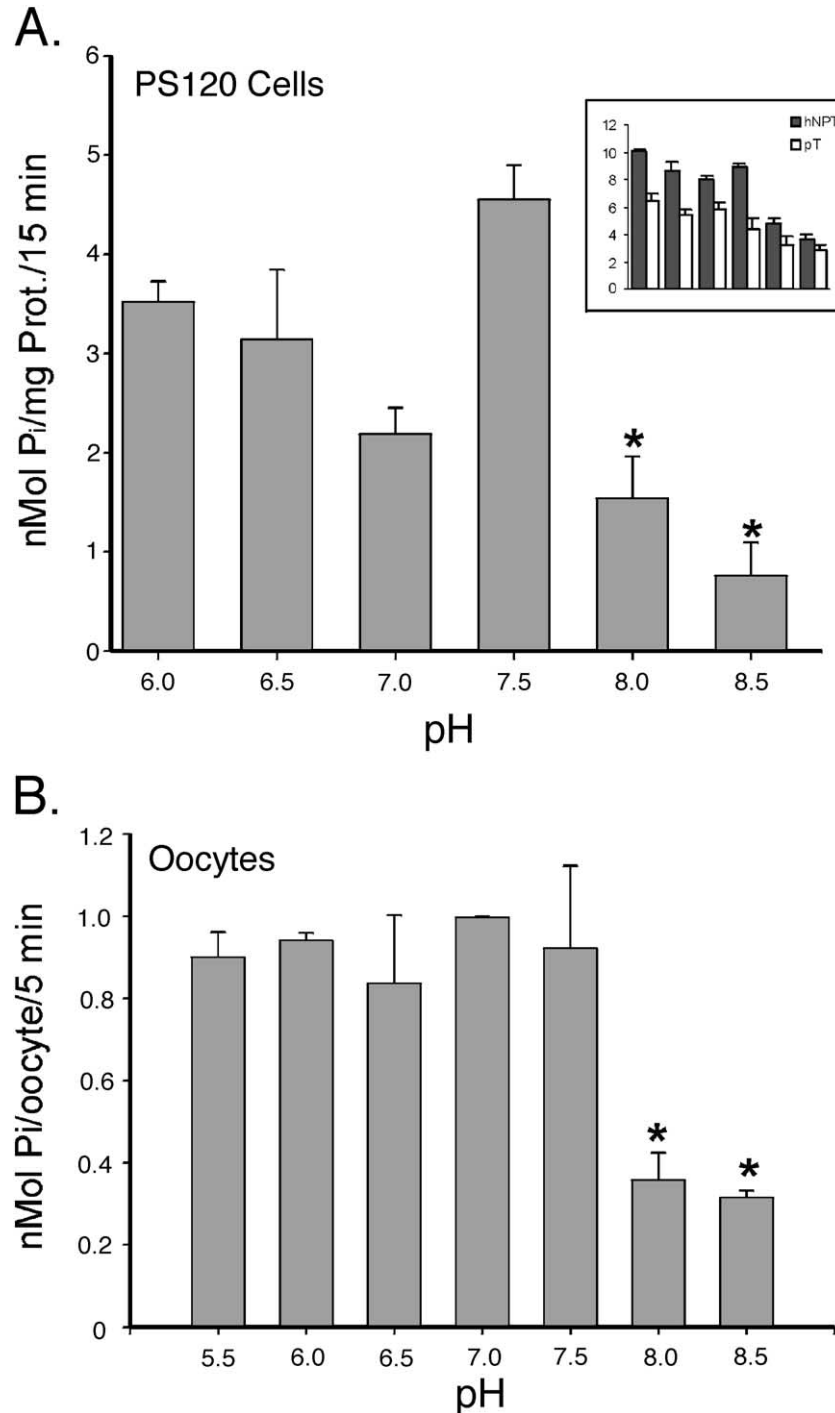


Fig. 5. Effect of pH on phosphate transport. Panel A. Phosphate uptake mediated by the human NaPi-IIb cotransporter in PS120 cells. Fifteen-minute uptake of 0.1 mM phosphate was measured in uptake solutions adjusted to pH values ranging from 6.0 to 8.5. The values shown are mean \pm S.E. ($n=5$; $*P<0.05$). The inset presents sodium-dependent Pi uptake in human NaPi-IIb cDNA (●) and vector DNA (○) transfected PS120 cells. The axis labels in the inset are the same as in the figure. Panel B. Phosphate transport in human NaPi-IIb cRNA injected oocytes. Five-minute uptake of 0.1 mM phosphate was measured in uptake solutions adjusted to pH values ranging from 5.5 to 8.5 in injected oocytes. The values shown are mean \pm S.E. ($n=10$ oocytes per pH group; $*P<0.01$).

3.4. Sodium kinetics of the human NaPi-IIb cotransporter

The effect of Na^+ ions on Pi transport mediated by the hNaPi-IIb cotransporter was explored by measuring phosphate transport as a function of Na concentration. Oocytes were injected with human NaPi-IIb cRNA, and PS120 cells were transfected with hNaPi-IIb cDNA or vector DNA. Na-dependent Pi uptake was measured in the presence of various sodium concentrations and a constant Pi concentration of 100 μM . There was a clear relationship between the initial rate of phosphate transport and the sodium concentration in hNaPi-IIb overexpressing PS120 cells, as well as in hNaPi-IIb cRNA-injected oocytes. The hNaPi-IIb protein generated a maximal velocity for Na^+ in PS120 cells of 4.38 nM/mg protein/12 min and the K_m was 34 mM. Similarly, in oocytes, the V_{\max} for Na^+ was 4.61 nM/oocyte/5 min and the K_m was 65 mM (Fig. 4B).

3.5. pH dependence of the human NaPi-IIb cotransporter

The effect of pH on human NaPi-IIb cotransporter activity was also tested in NaPi-IIb cDNA transfected PS120 cells and in oocytes. The pH range tested was from 6.0 (where Pi is monobasic) to 8.5 (where Pi is dibasic) [22]. The sodium-dependent Pi uptake mediated by the human NaPi-IIb cotransporter remained relatively constant at pH values between 6.0 and 7.5, but was significantly inhibited at alkaline pH levels (Fig. 5A). Similar results were also observed in hNaPi-IIb cRNA-injected oocytes (Fig. 5B).

3.6. Substrate specificity of the hNaPi-IIb cotransporter in stably transfected PS120 cells

To further explore substrate selectivity, a variety of compounds, including phosphate, glycine, glucose, alanine, ascorbic acid and sulfate, were tested in hNaPi-IIb cDNA transfected PS120 cells. The results showed that radiotracer phosphate uptake mediated by the human NaPi-IIb protein was not affected by 100-fold excesses (10 mM) of glycine, glucose, alanine and ascorbic acid, but was slightly inhibited ($\sim 30\%$) by sulfate in PS120 cells (data not shown).

3.7. Inhibition study of the hNaPi-IIb cotransporter in stably transfected PS120 cells

Arsenate, PFA and PMA have been previously shown to inhibit sodium-dependent phosphate uptake [23–26]. Therefore, we tested the sensitivity of the human NaPi-IIb cotransporter to these compounds. PS120 cells were transfected with vector DNA or hNaPi-IIb cDNA, and sodium-dependent Pi uptake was measured in the presence or absence of these potential inhibitors. As shown in Fig. 6, PFA (5 mM) inhibited nearly all of the phosphate uptake mediated by the human NaPi-IIb transporter, while arsenate (1 mM) only inhibited $\sim 60\%$ of the phosphate uptake (Fig. 6A). PMA at a concentration of 100 nM blocked most

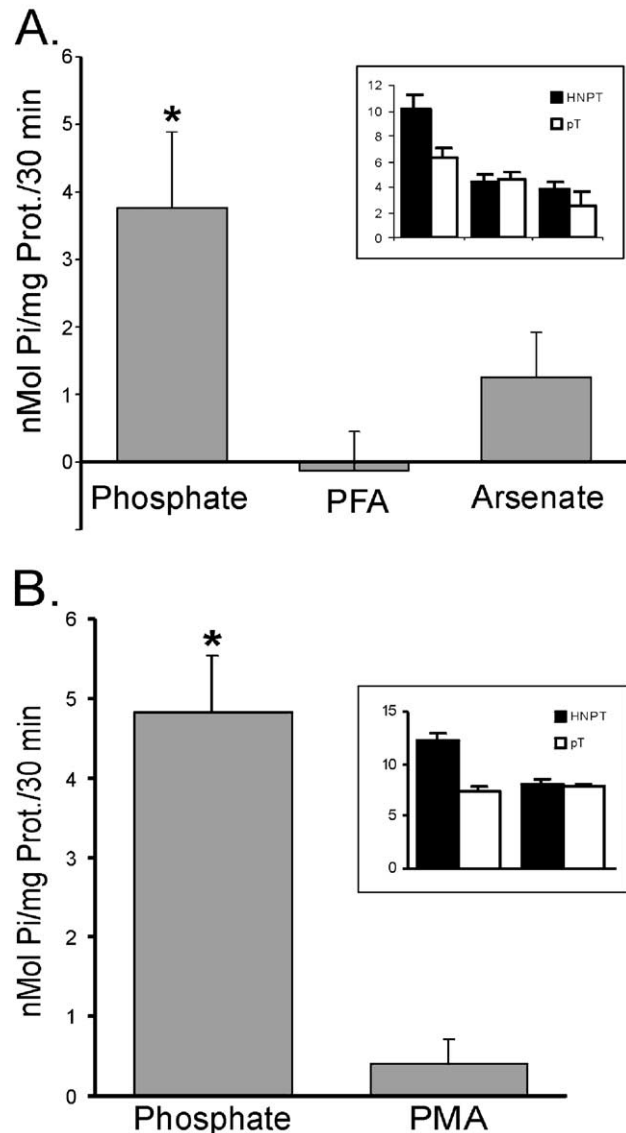


Fig. 6. Inhibition study of sodium-dependent phosphate uptake mediated by hNaPi-IIb in PS120 cells. Uptake was measured in uptake solutions containing various known NaPi cotransport inhibitors in PS120 cells. The values shown are mean \pm S.E. ($n=6$; $P<0.02$). The insets present the sodium-dependent Pi uptake in hNaPi-IIb cDNA (●) and vector DNA (○) transfected cells. The axis labels in the insets are the same as in the figures. Panel A shows the results in the presence of 5 mM PFA or 1 mM arsenate. Panel B highlights the results in the presence of 100 nM PMA.

of the phosphate uptake mediated by the human NaPi-IIb cotransporter (Fig. 6B).

4. Discussion

PS120 cells are fibroblast cells derived from lung tissue of Chinese hamsters [11]. These cells have been used to express several intestinal transport proteins, including several sodium–hydrogen exchangers [14,16,17,19]. These studies suggested that PS120 cells are a suitable system to

functionally express intestinal transport proteins. Therefore, we stably expressed the recently cloned human intestinal sodium-dependent phosphate cotransporter (NaPi-IIb) in PS120 cells by liposome-mediated transfection, followed by G418 selection. The sodium-dependent phosphate uptake in hNaPi-IIb cDNA transfected PS120 cells significantly increased compared with uptake in vector DNA transfected cells. This indicates that PS120 cells can functionally express the human NaPi-IIb cotransporter, which now makes it possible to study transport characteristics and functional regulation of the human NaPi-IIb cotransporter in mammalian cells. The hNaPi-IIb cotransporter expression level in PS120 cells is modest, and this may be due to the toxicity of high intracellular phosphate concentrations introduced by overexpression of the human NaPi-IIb cotransporter protein [27–29].

Functional characterization of the human NaPi-IIb cotransporter was performed in stably transfected PS120 cells and in the oocyte expression system. The results obtained from these studies showed that the sodium-dependent phosphate transport observed in transfected PS120 cells was similar to the activity observed in oocytes, with respect to K_m for sodium (34 mM in PS120 cells and 65 mM in oocytes) and K_m for phosphate (106 μ M in PS120 cells and 112 μ M in oocytes). The uptake mediated by hNaPi-IIb was also pH-dependent, and was inhibited at alkaline pH levels. The kinetic parameters determined in both transfected PS120 cells and oocytes indicate that the human NaPi-IIb cotransporter does not have a high affinity for phosphate, and that its activity is regulated by pH. Furthermore, the phosphate and sodium kinetics of the human NaPi-IIb cotransporter observed in oocytes is similar to the kinetics observed from the mouse NaPi-IIb cotransporter, although the mouse NaPi-IIb cotransporter was not inhibited at alkaline pH values in oocytes [30].

Substrate selectivity studies showed that sulfate slightly inhibited phosphate uptake mediated by the human NaPi-IIb in PS120 cells, which suggests that this cotransporter may be involved in sulfate uptake in the intestine. However, further studies are necessary before strong conclusions may be drawn.

Previous studies have shown that arsenate and PFA could inhibit phosphate absorption mediated by NaPi cotransporters [21,23,26,31,32]. Our studies also showed that these compounds significantly inhibited sodium-dependent phosphate absorption mediated by the human NaPi-IIb cotransporter. Interestingly, PFA only inhibited a small portion (~29%) of endogenous sodium-dependent phosphate uptake in vector DNA transfected PS120 cells, while it inhibited ~55% of sodium-dependent phosphate transport in hNaPi-IIb cDNA transfected PS120 cells (Fig. 5, insert). This finding suggests that the human NaPi-IIb cotransporter is more sensitive to PFA than the endogenous sodium-dependent phosphate uptake system present in PS120 cells.

Protein kinase C (PKC) activation has been shown to be involved in the regulation of renal Na-dependent

phosphate absorption by parathyroid hormone (PTH) [33,34]. PMA, a potent activator of PKC, inhibited sodium-dependent Pi uptake in mouse kidney [33] and opossum kidney (OK) cells [35], as well as in NaPi-IIa cRNA-injected oocytes [36,37]. PMA also inhibited NaPi-IIb cotransporter activity in oocytes [37]. In our PS120 cell expression system, similar results were observed. 100 nM PMA abolished phosphate uptake mediated by the human NaPi-IIb cotransporter, but had no effect on the endogenous NaPi cotransporter system. Therefore, these data suggest that the hNaPi-IIb cotransporter is inhibitable by PKC activation in mammalian cells. Further studies will be required to decipher the molecular mechanism of this regulation.

The kinetic characterization in vector DNA transfected PS120 cells suggested that another NaPi cotransporter(s) is expressed in these cells (as shown in the figure inserts). This observed activity is likely due to the presence of type III NaPi cotransporters, which are ubiquitously expressed in mammalian cells [21]. This type III NaPi cotransporter was also inhibited by PFA, arsenate and alkaline pH. However, overexpression of the hNaPi-IIb cDNA in PS120 cells led to a significant increase in Na-dependent Pi cotransport over background, and this increase could be completely blocked by 5 mM PFA and 100 nM PMA.

In summary, this study characterized the human intestinal NaPi-IIb cotransporter in stably transfected PS120 cells and in the oocyte expression system. Both PS120 cells and oocytes were able to functionally express the hNaPi-IIb cotransporter, which mediated pH-dependent NaPi cotransport. PFA, PMA and arsenate inhibited the phosphate transport mediated by the human NaPi-IIb cotransporter in PS120 cells. We conclude that the PS120 cell expression system might be useful to identify specific inhibitor(s) of type IIb NaPi cotransporter.

Acknowledgements

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